A Cellular Timetable of Autumn Senescence[1]

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We have studied autumn leaf senescence in a free-growing aspen (*Populus tremula*) by following changes in pigment, metabolite and nutrient content, photosynthesis, and cell and organelle integrity. The senescence process started on September 11, 2003, apparently initiated solely by the photoperiod, and progressed steadily without any obvious influence of other environmental signals. For example, after this date, senescing leaves accumulated anthocyanins in response to conditions inducing photooxidative stress, but at the beginning of September the leaves did not. Degradation of leaf constituents took place over an 18-d period, and, although the cells in each leaf did not all senesce in parallel, senescence in the tree as a whole was synchronous. Lutein and β-carotene were degraded in parallel with chlorophyll, whereas neoxanthin and the xanthophyll cycle pigments were retained longer. Chloroplasts in each cell were rapidly converted to gerontoplasts and many, although not all, cells died. From September 19, when chlorophyll levels had dropped by 50%, mitochondrial respiration provided the energy for nutrient remobilization. Remobilization seemed to stop on September 29, probably due to the cessation of phloem transport, but, up to abscission of the last leaves (over 1 week later), some cells were metabolically active and had chlorophyll-containing gerontoplasts. About 80% of the nitrogen and phosphorus was remobilized, and on September 29 a sudden change occurred in the $\delta^{15}N$ of the cellular content, indicating that volatile compounds may have been released.

Autumnal senescence may attract more attention from the public, but less from scientists, than any other plant developmental process. Every autumn deciduous trees need to shed their leaves and prepare for the winter. Autumnal senescence is spectacular in many trees, when leaves change color from green to yellow and/or red (Lee et al., 2003). The different colors arise from the preferential degradation of chlorophylls over carotenoids and the synthesis of red-colored pigments like anthocyanins (Goodwin, 1958; Lichtenthaler, 1987).

Autumn senescence, like other forms of leaf senescence, is a type of programmed cell death, i.e. the leaf cells die in an organized, predetermined way controlled by the nucleus. On the other hand, there are fundamental differences between the leaf senescence program and apoptotic processes, which have been studied in great detail in animal systems and more recently in plants (Noodén et al., 1997; Kuriyama and Fukuda, 2002). In fact, it has even been speculated that the processes are mutually antagonistic (Ougham et al., 2005) since an appropriately executed senescence program avoids the pathological consequences of cell death (Hörtensteiner, 2004). Senescence has adaptive value because of the associated remobilization of nutrients, especially nitrogen, and, to a lesser extent, phosphorus, sulfur, and other elements (Himelblau and Amasino, 2001; Hörtensteiner and Feller, 2002). The timing of autumn senescence can be regarded as the result of a trade-off between the conflicting requirements for optimizing the nitrogen and carbon status of the plant. Trees entering senescence early will efficiently remobilize nitrogen at the expense of photosynthetic yield, while trees entering senescence late will gain more photosynthates, but in some years their leaves will die before the remobilization of their nutrients is complete. Nitrogen status influences the onset of autumn senescence. For instance, alders (*Alnus* subsp.) that host a nitrogen-fixing symbiont (Frankia) shed their leaves while they are still green, and gardeners in temperate regions know that nitrogen-rich fertilization should be avoided since it delays both autumn senescence and the development of hardiness, thereby compromising winter survival (especially of woody perennials).

Autumn senescence has not been well characterized at the cellular and molecular levels, but more information is available regarding leaf senescence in annuals (Buchanan-Wollaston, 1997; Quirino et al., 2000). The chloroplasts, present in green leaves, differentiate into gerontoplasts that lack stacked thylakoid membranes but are rich in electron-dense lipid bodies, plastoglobuli, which have an especially high content of carotenoids and carotenoid esters (Tevini and Steinmuller, 1985). Nutrient remobilization requires the breakdown of macromolecules and conversion of the breakdown products into transportable compounds. Many genes coding for catabolic enzymes (proteases, lipases, nucleases, etc.) are among those that are induced during senescence (Bhalerao et al., 2003; Buchanan-Wollaston

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et al., 2003; Andersson et al., 2004; Guo et al., 2004). When the photosynthetic capacity of the leaves is lost, the energy required for the remobilization must be provided by mitochondrial respiration and, consequently, nuclear genes coding for components of the mitochondrial electron transport chain are not downregulated during autumn senescence, in contrast to genes coding for components of the photosynthetic apparatus (Buchanan-Wollaston, 1997; Andersson et al., 2004). Although the leaves do not perform photosynthesis, phloem transport out of the leaf is needed to export the nutrients so, from a transport perspective, senescing leaves are still source leaves. Eventually, however, phloem transport stops, a protective layer is formed on the inner side of the petiole, a separation or abscission layer is formed proximal to it, and when the cell walls in the separation layer are gradually loosened, the leaf eventually falls (Roberts et al., 2002). It is well known that autumn senescence in most trees is triggered by reductions in the photoperiod, a signal that is more reliable than temperature as a harbinger of the first strong frost, allowing autumn senescence calendars to be constructed that are quite constant over the years (e.g. www.great-lakes.net/tourism/fallcolor.html; www.arkansas.com/calendar/fall_foliage_pg1.asp). The pivotal role of phytochromes in the process has also been well documented (Olsen et al., 1997; Chen et al., 2002), but the extent to which low temperatures, perhaps coupled to light intensities inducing photooxidative stress, modulate the process is not clear. It is common knowledge that cold autumns result in intense leaf coloration, but the physiological basis of this color change is not well understood.

We have started a project to elucidate the genetic basis of autumn senescence in aspen (*Populus tremula*), and as part of the project we have identified many genes that are expressed in autumn leaves (Bhalerao et al., 2003) and constructed a transcriptional timetable for autumn senescence (Andersson et al., 2004). However, studies of gene expression alone do not always provide sufficient information for understanding the cellular events that occur during the senescence process, especially since not only anabolism, but also gene expression, ceases during the later stages of autumn senescence (Andersson et al., 2004). Consequently, catabolic processes leading to the degradation of cellular components and, eventually, cell death should perhaps be studied at the cell biological level rather than the transcriptional level.

Many fundamental questions concerning cellular processes in autumn leaves in aspen, and trees in general, have not yet been answered, or in some cases even addressed. For instance, is the conversion of chloroplasts to gerontoplasts manifested by the appearance of autumn colors driven only by developmental factors, or do environmental stress factors like photooxidation also contribute? When and to what extent are the nutrients of the leaf remobilized? When has photosynthesis decreased to the point that mitochondrial respiration has to provide the energy required for the degradation process and, after this point, do the plastids still play a role in the remobilization of nutrients? When, and how, are the gerontoplasts, nuclei, and mitochondria degraded, and when does the cell eventually undergo cell death? Finally, what are the signals that trigger the onset of autumn senescence? To address these questions, we have monitored the timing of autumn senescence events in a free-growing aspen, and here present a cellular timetable for them.

RESULTS

Autumn Senescence Progressed through Four Phases

As reported for the same tree in the autumn of 1999 (Bhalerao et al., 2003), autumn senescence in 2003 largely progressed in a synchronized fashion. Autumn colors appeared earlier in some leaves than in others, and patches of each leaf turned yellow before others, but there were no apparent differences between the different parts of the tree in these respects (Fig. 1, A and B). This is typical for aspen trees, but not for all other tree species. Over the years, we have visually examined thousands of aspens, but we have found no cases in which one part of the tree had progressed further into autumn senescence than others. In contrast, maples (*Acer* subsp.), for example, frequently have autumn coloration in the part of the crown that is most exposed to sunlight, while the leaves are still green in other parts. Yet other species may have green shoot tips, while the inner parts are yellow. Such differences, together with differences in leaf color, often allow tree species to be recognized from long distances (Chapman et al., 2000). This feature makes aspen a convenient model system for studies of autumn senescence since sampling and pooling randomly selected leaves are likely to give results that are representative for the whole tree.

The weather conditions during the experimental period, recorded by a weather station about 200 m from the tree, are displayed in Figure 1B. After a few cold days with bright sun (August 31 to September 2), the conditions were very stable, with relatively mild, clear days up to September 19, when the temperature fell sharply. The chlorophyll content of the leaves (Fig. 2A), expressed on a dry-weight basis, was more or less stable up to September 11. Concentrations of both chlorophyll *a* and *b* started to decrease on September 11 (Fig. 2A). The chlorophyll *a/b* ratio was constant until September 24, after which it decreased for about 3 d, stabilized again, and finally decreased in phase 4. The minor variations observed in chlorophyll concentration before September 11 could have been due to inhomogeneity in the sampled leaf material. In a free-growing tree, fungal infections, and to some extent insect herbivory, induce patches of senescence in the leaf. These are stochastic events; note that the standard deviation of the measurements of chlorophyll concentration are larger up to the point where chlorophyll degradation due to autumn senescence started, apparently overruling these leaf-to-leaf differences. However,

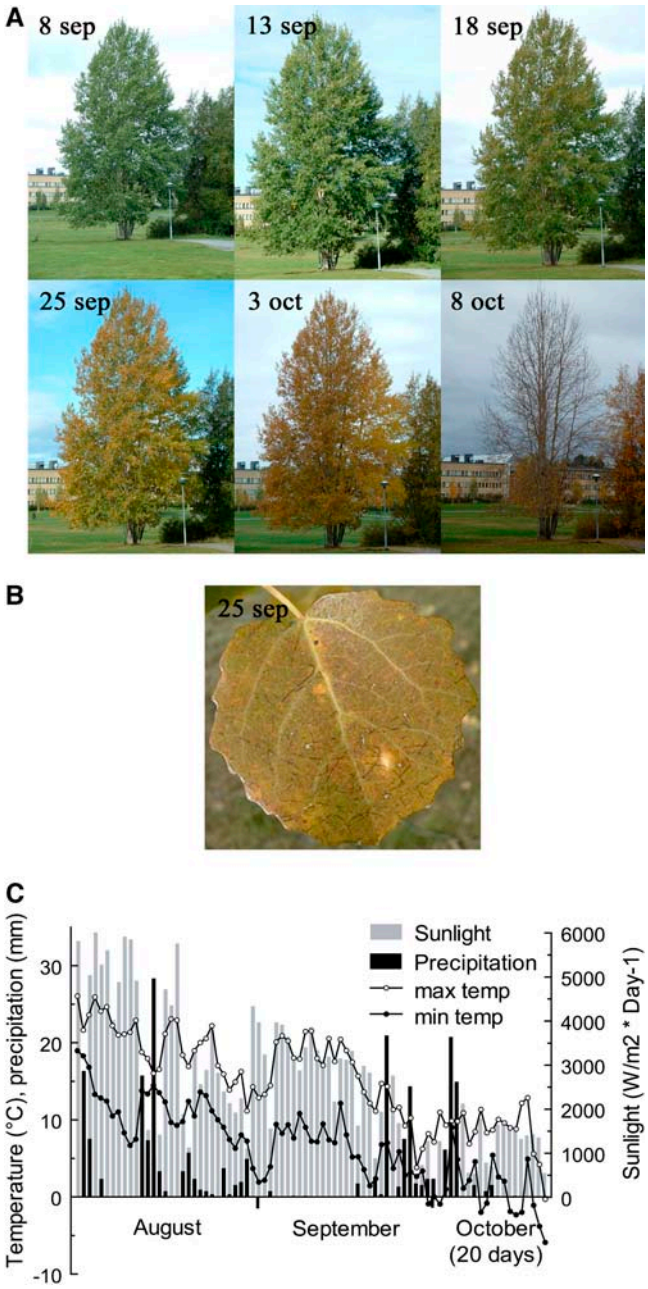

Figure 1. A, Autumn senescence in a free-growing aspen. The images were taken at the time of leaf sampling (11:30 AM) during the autumn of 2003. B, Senescing aspen leaf (September 25). C, Weather conditions during the sampling period. Gray bars represent the amount of sunlight (W/m^2) per day and black bars represent the millimeters of precipitation per day. The lines correspond to the maximum and minimum temperatures for each day. sep, September; oct, October.

it is possible that the dip in chlorophyll concentration observed around September 1 was a consequence of the cold, sunny weather at that time, inducing photooxidative stress and some photobleaching. If so, the recovery of chlorophyll levels in the first week of September indicates that the leaves were still not fully prepared to undergo autumn senescence. Whether or not this is true, the time up to September 11, when chlorophyll levels were stable, clearly represents a presenescent stage, which we designated phase 1. After September 11, chlorophyll levels started to decrease (phase 2) at a constant rate until the last days of September, when chlorophyll concentration (on a dry-weight basis) had decreased to about 10% of phase 1 levels. In phase 3, little or no further degradation of chlorophyll occurred, but a few days before abscission, starting on about October 6, there may have been a small further decrease in chlorophyll content (phase 4).

Degradation of leaf components leads, of course, to the loss of leaf biomass. In order to monitor the changes in leaf biomass over the period, we calculated leaf dry weights of five randomly chosen leaves each day during the studied period. Although differences in the size of the five leaves resulted in fluctuations in the dry-weight data (Fig. 2B), there was an overall tendency for average leaf weight to start declining, from an initial value of around 150 mg, at about the same time as chlorophyll levels (about September 11), reaching a final value of about 75 mg. On average, leaf dry weight fell by 40% to 50%, showing that roughly

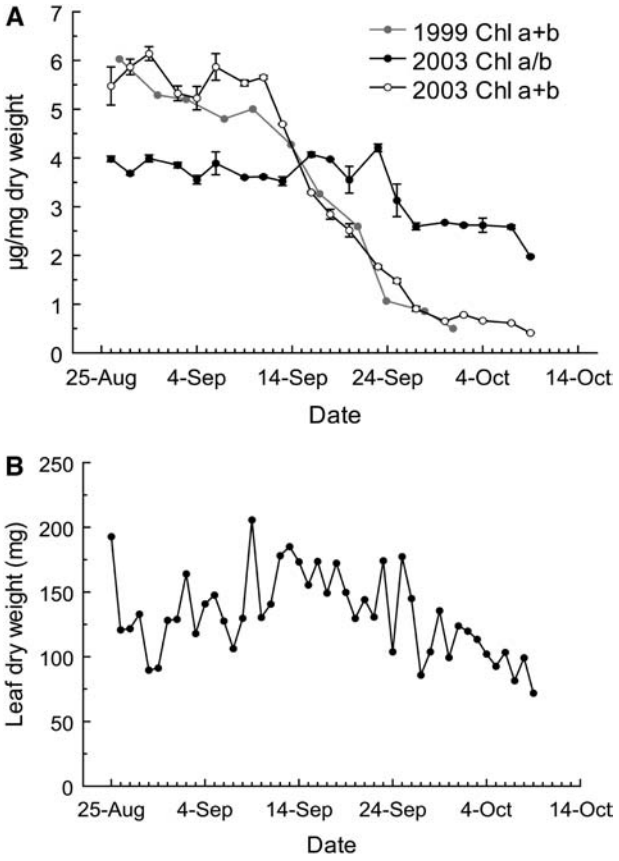

Figure 2. A, Chlorophyll concentrations in aspen autumn leaves. White and black circles represent total chlorophyll ($a + b$) for 2003 and the chlorophyll a/b ratio for 2003, respectively. Each point is the mean of three measurements (±SD). The gray background line represents total chlorophyll ($a + b$) for 1999 (as trend comparison, not actual values). B, Leaf weight (dry). Five leaves were pooled, ground, and weighed, and then dry leaf weight was calculated from the weight loss (%) after drying a small amount of the material.

50% of the dry mass had been remobilized from the leaves. This resulted in chlorophyll levels in phase 3, per leaf, being about 5% of phase 1 values. It also seems that some chlorophyll degradation occurred in phase 3, since the average leaf dry weight decreased slowly, while the amount of chlorophyll per unit dry leaf weight was constant.

In order to compare the progression of senescence in 2003 with the transcriptional timetable of autumn senescence that we generated for the same tree for the autumn of 1999, the chlorophyll concentrations in the leaves used for RNA preparation in 1999 were calculated and are presented in Figure 2A. The dates of the transitions between phases 1, 2, and 3 were almost identical in the two years. Our separation of the autumn senescence process into four phases provides a framework for the following examination of the cellular timetable for autumn senescence.

The fact that chlorophyll degradation was initiated on the same date in both years indicates that the photoperiod was the dominant trigger for the transition into phase 2, since the weather conditions in the 10 d preceding September 11 were very stable and mild in both 2003 (Fig. 1B) and 1999 (data not shown). However, in both years, the days around September 1 were unusually cold but sunny, and these conditions could have triggered entry into phase 2, following a 10-d delay. To prove or disprove this hypothesis, we also examined chorophyll concentrations in the leaves sampled in 2001 and correlated them with the weather conditions (data not shown). In this year, the days around September 1 were not unusually cold, but chlorophyll degradation was nevertheless initiated around September 11, indicating that the tree initiated autumn senescence in response solely to changes in the photoperiod.

Dynamic Changes in Carotenoid and Anthocyanin Metabolism

To establish whether the yellow autumn color in these leaves depends on synthesis of new carotenoids or their slower degradation, we also measured their carotenoid content and composition throughout the period using HPLC. Figure 3A shows the levels of neoxanthin, lutein, β-carotene, and the xanthophyll cycle pigments. The total carotenoid concentration started to decrease at the same time that chlorophyll degradation started, during the transition from phase 1 to phase 2. However, the carotenoid degradation rate was slower, resulting in the color shift of the leaves from green to yellow. At the time when the leaves appeared yellow, the chlorophyll levels had decreased by 75%, but carotenoid levels only by about 50%. In phase 3, carotenoid levels were quite stable, but in phase 4 they again started to decrease. There were significant differences in degradation rates between the different carotenoids. β-Carotene was degraded largely in parallel to chlorophyll; the degradation rate for lutein also followed the four-phase pattern, although the levels did not decrease as much as for chlorophyll. Neoxanthin

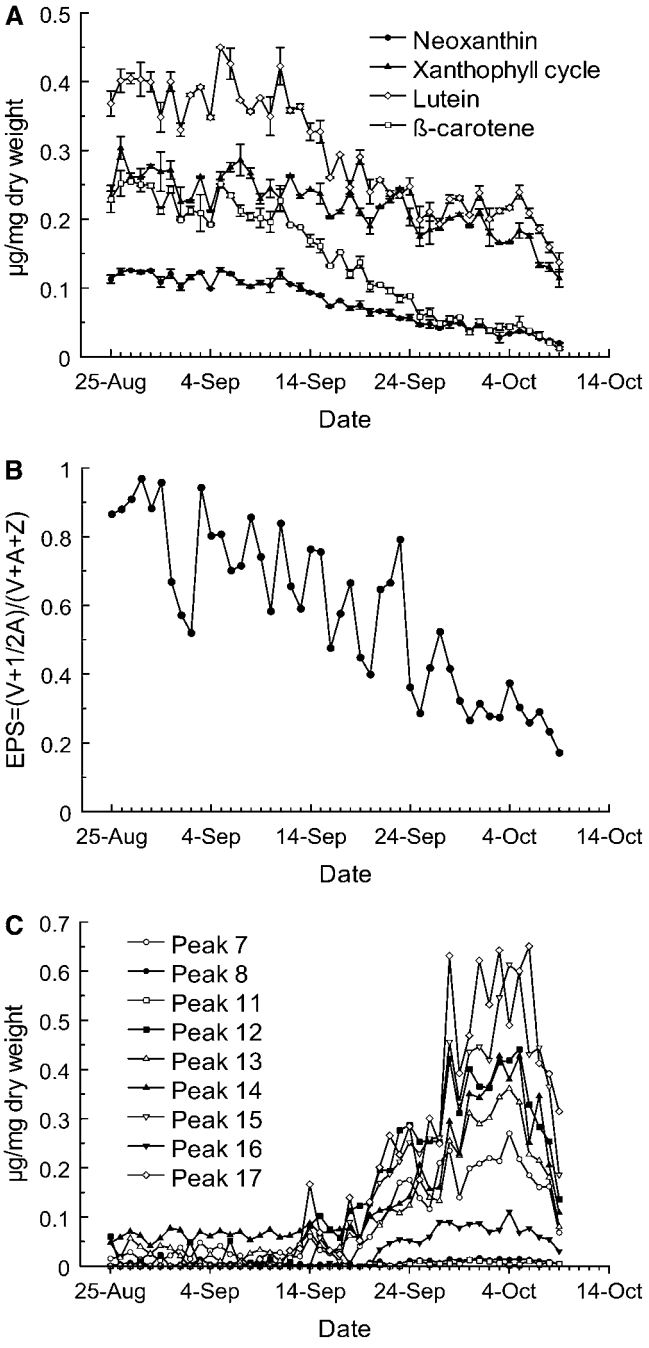

Figure 3. Carotenoid concentrations in aspen autumn leaves. A, Total carotenoid content. Symbols refer to neoxanthin (circles), xanthophylls (triangles), lutein (diamonds), and β-carotene (squares). Each point is the mean value of two measurements (±SD). B, Epoxidation state of xanthophyll cycle pigments. C, Accumulation of carotenoid degradation products. Lines correspond to the different peaks in Table I.

levels roughly followed chlorophyll levels throughout phase 2, but also continued to decrease in phases 3 and 4. The total concentration of the xanthophyll cycle pigments (violaxanthin, antheraxanthin, and zeaxanthin) were more stable and decreased only by about 50% on a dry-weight basis (75% on a per leaf basis) during the studied period.

The xanthophyll cycle pigments can be interconverted in the xanthophyll cycle, and in green leaves the dynamics of the xanthophyll pools are determined by environmental factors. Violaxanthin is converted to zeaxanthin via antheraxanthin in a reaction catalyzed by the enzyme violaxanthin de-epoxidase when the lumenal pH decreases as a consequence of the light reactions, producing a higher proton gradient than can be utilized in CO_2 fixation (Demmig-Adams, 1990). Such weather-dependent changes in the xanthophyll pools were clearly observed until the end of phase 2 (Fig. 3B). However, even throughout phase 3, such changes occurred, although the amplitude was greatly reduced, and the changes observed were perhaps not significant.

From the middle of phase 2, new peaks started to appear in the chromatograms (Suzuki and Shioi, 2004). We could distinguish nine pigments in addition to the normal photosynthetic pigments, which accumulated during autumn senescence (Fig. 3C). These carotenoids/carotenoid derivatives were tentatively identified by recording their absorbance spectra and by exposing the extracts to high pH (saponification), a treatment that breaks the ester bond of carotenoid esters and releases the carotenoid moieties (Granado et al., 2001). To our knowledge, these pigments have not been thoroughly characterized before, and we did not attempt to rigorously do so here; thus, the assignments are only tentative (Table I; for details of the tentative identification, see ''Materials and Methods''). Although some of these pigments (CX, NE2, NE3, and VE1) were already present in small amounts in phase 1, accumulation started in phase 2 (on September 12, 15, and 21 for VX, NE4, and VE2, respectively). The pattern for CY and NE1 was not clear, but accumulation seemed to start around September 20. Formation of the first, as-yet uncharacterized carotenoid catabolites appeared to coincide with the onset of chlorophyll breakdown, but several of them did not appear until later stages of senescence when chlorophyll levels had decreased by 50% or more. During phase 4, these carotenoid catabolites decreased in parallel with the decrease in other carotenoids.

Table I. *Photosynthetic pigments and pigment catabolites in aspen autumn leaves, separated by HPLC*

Several of the peak assignments are tentative (see ''Materials and Methods'' for details).

Peak	Retention Time	Compounds
	min	
1	1.97	trans-Neoxanthin
2	2.14	9-cis-Neoxanthin
3	2.59	Violaxanthin
4	3.71	Antheraxanthin
5	5.15	Lutein
6	5.44	Zeaxanthin
7	6.19	Carotenoid X (CX)
8	6.51	Carotenoid Y (CY)
9	7.71	Chlorophyll *b*
10	8.00	Chlorophyll *a*
11	8.18	Neoxanthin ester 1 (NE1)
12	8.30	Neoxanthin ester 2 (NE2)
13	8.50	Violaxanthin ester 1 (VE1)
14	8.86	Neoxanthin ester 3 (NE3)
15	9.40	Neoxanthin ester 4 (NE4)
16	9.64	Violaxanthin ester 2 (VE2)
17	10.00	Violaxanthin X (VX)
18	10.73	β-Carotene

Although these pigments accumulated strongly during autumn senescence, none of them seemed to become the dominant pigments in the autumn leaves. We do not know their exact extinction coefficients so they could not be accurately quantified, but since saponification appeared to convert them quantitatively into neoxanthin, violaxanthin, and antheraxanthin, the increases in the concentrations of these pigments after saponification is likely to represent the amounts of the corresponding carotenoid esters. On October 3, neoxanthin, violaxanthin, and antheraxanthin collectively accounted for about 25% of the total carotenoid pool (including lutein, zeaxanthin, and β-carotene), and since the amount of these pigments increased by about 50% upon saponification, we estimate that the total amount of esterified carotenoids corresponded to 10% to 15% of the total carotenoid pool.

Carotenoids are not the only pigments responsible for autumn colors; anthocyanins often accumulate (Lee et al., 2003), giving rise to dark-reddish leaf colors, and have been proposed to function as light protectants during the nutrient recycling of senescing leaves (Feild et al., 2001; Hoch et al., 2003). Therefore, we quantified anthocyanin levels to see whether they changed during autumn senescence in aspen leaves. Spectroscopic measurements of the total anthocyanin concentration in extracts of the leaves showed that they did indeed increase during autumn senescence (Fig. 4). The levels were low and constant up to about 5 d into phase 2 (September 15), at which point chlorophyll levels had decreased by about 25%. The onset of anthocyanin accumulation was not apparently correlated to photooxidative stress, since the 12 d preceding September 15 were all sunny but very mild. However, once anthocyanin accumulation had been initiated, further accumulation seemed to be strongly dependent on excess light conditions, i.e. cold and sunny weather. The clear, cold days on September 19 and 20 induced a huge accumulation of anthocyanins, more than one-half of which disappeared in the milder and rainy week that followed, while frost and sun on September 30 resulted in a new accumulation, followed by degradation during the mild and rainy days at the beginning of October. Finally, from October 4 onward, the weather was clear and cold, resulting in a very high accumulation of anthocyanins.

Leaves Lose Photosynthesis Activity and Energy Requirements Are Then Met by Mitochondria

When the chlorophyll starts to be degraded and the chlorophyll-binding proteins are remobilized, the

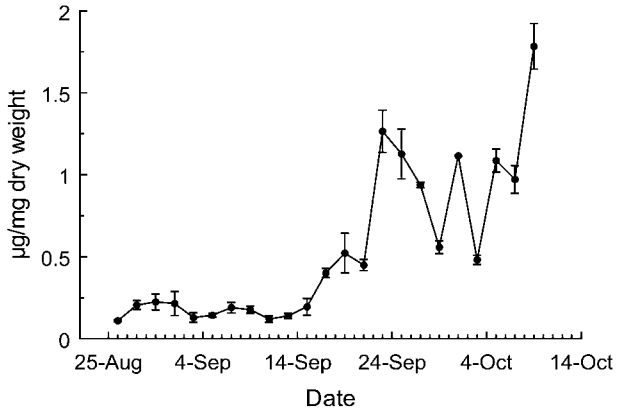

Figure 4. Anthocyanin concentrations in aspen autumn leaves. Each point is the mean value of two measurements (±SD).

photosystems are affected and photosynthesis declines. Although chlorophyll levels reflect the overall amounts of chlorophyll-binding proteins, they provide no direct indication of whether or not the photosynthetic apparatus is functional, so we wanted to directly estimate the photosynthetic activity in autumn leaves. Performing accurate gas exchange measurements on attached branches directly in the field was not feasible, and detached branches lost their photosynthetic capacity quite rapidly, presumably due to stomatal closure. The most convenient way is to measure chlorophyll fluorescence, which provides a sensitive tool for studying photosynthetically active PSII centers in vivo. In healthy, unstressed leaves, the F_v/F_m ratio is 0.80 to 0.85, whereas in stressed leaves, the ratio can decrease to 0.70 or less (Öquist and Wass, 1988). However, when interpreting the F_v/F_m ratio data, it must be remembered that they are relative measures that reflect the status of the PSII centers present and do not provide accurate quantifications of the number of centers.

We followed four leaves during the autumn and measured F_v/F_m twice a week (Fig. 5). One leaf appeared to be significantly stressed at the beginning of the measurement series and showed F_v/F_m values below 0.6. However, this leaf recovered and its values became similar to those of the other leaves, showing that at least up to the first week of September, the PSII repair capacity was sufficient to restore damaged PSII centers. Somewhat surprisingly, F_v/F_m ratios stayed high and more or less constant until September 26, when chlorophyll concentrations had decreased by about 80%, i.e. throughout almost all of phase 2. Apparently, therefore, the remaining PSII centers were photosynthetically active even very late in the senescence program. Since a wide variety of stresses that affect either the chloroplast or the rest of the cell can influence F_v/F_m, this finding shows that degradation of the photosynthetic apparatus was tightly regulated. At the very end of phase 2 and later in phase 3, F_v/F_m declined in all leaves, but a measurable F_v/F_m ratio was present in one of the leaves even on October 3, indicative of active PSII centers.

When the photosynthetic capacity decreases, mitochondria must presumably take over as the main energy sources when the chloroplasts and other cell components are degraded. To examine this assumption, we measured ATP and ADP levels in leaves that were instantly frozen in liquid nitrogen in the light (Fig. 6A).

The adenylate content (ATP + ADP) was constant during phase 1 and the ATP/ADP ratio was initially relatively low (around 2), but gradually increased to almost 4. During phase 2, the content of adenylates decreased in a very similar way to chlorophyll and, during the first part of phase 2, the ATP/ADP ratio rapidly increased from 4 to about 8. The decrease in adenylate content may reflect degradation of chloroplasts, which contain most of the cellular adenylate pool (Gardeström and Wigge, 1988). Also, the chloroplast compartment typically has a lower ATP/ADP ratio than the rest of the cell (Gardeström and Wigge, 1988; Bykova et al., 2005). Thus, the observed increases in ATP/ADP ratios are consistent with degradation of the chloroplast pool.

Taken together, this evidence shows that chloroplasts are the major energy sources for the leaves in phase 1. In phase 2, the degradation of the photosynthetic apparatus creates a gradual shift toward an increasing dependence on mitochondrial respiration, and from the middle of phase 2 (September 19) and throughout phase 3, mitochondria seem to provide most of the autumnal leaves' energy. In phase 4, the drop in ATP/ADP ratios and the total adenylate pool also indicates that remaining mitochondria are being degraded.

To get further insight into this transition from an organ dependent on photosynthesis to an organ dependent on respiration, we also wanted to determine the time at which the cells stopped accumulating sugars and starch. Sugars (Suc, Glc, and Fru) and starch were measured in a combined assay from the pool of leaves that was also used for adenylate analysis. During phase 1, Suc and starch were the major components, and very low amounts of Glc and Fru were detected. In

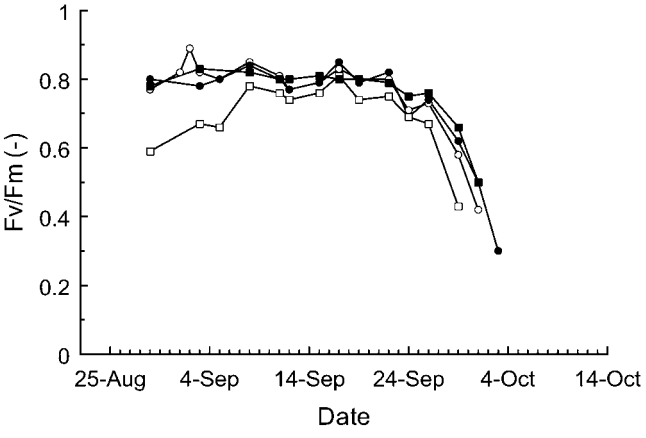

Figure 5. Photochemical efficiency of PSII (F_v/F_m) in aspen autumn leaves. Four leaves were followed during the autumn.

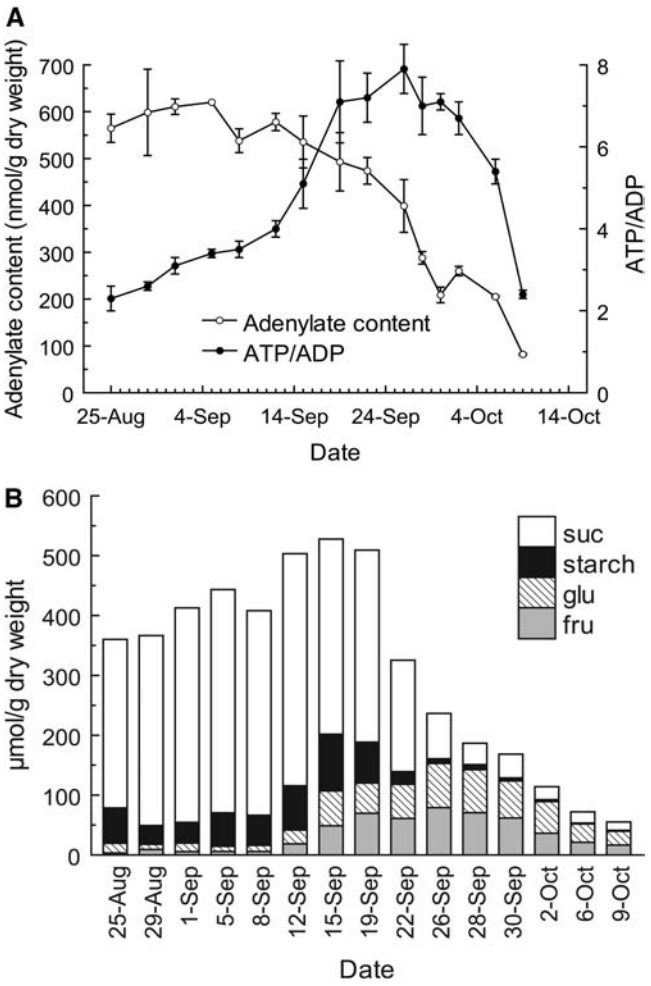

Figure 6. Changes in energy status and sugar depletion in aspen autumn leaves. Each point is the mean value of three measurements. A, Adenylate content (ATP + ADP) and ATP/ADP ratio. B, Concentrations of soluble sugars and starch.

phase 2, Glc and Fru levels initially increased, while Suc and starch were still abundant. Consequently, the highest content of total carbohydrates was observed on September 15, when chlorophyll had already started to decrease. After that, Suc and starch rapidly decreased so that, after September 22, Glc and Fru were the dominating sugars, staying at high levels throughout phase 2, before decreasing during phases 3 and 4.

Nitrogen and Phosphorus Are Remobilized in Aspen Autumn Leaves

To obtain data on net fluxes of nutrients from the autumn leaves, we subjected leaves to elementary analysis, measuring $\delta^{13}C$ and $\delta^{15}N$ and levels of carbon, nitrogen, potassium, sulfur, phosphorus, and iron in the leaves during the senescence process. The $^{13}C/^{12}C$ ratio did not change significantly (data not shown) but, as shown in Figure 7A, the relative content of carbon and nitrogen and the $\delta^{15}N$ value changed substantially during the autumn. On a dry-weight basis, the carbon content was constant, which is not surprising since carbohydrates account for the vast majority of leaf biomass. Since the average weight of the leaf decreased by about 50% over the time period, it seems that about 50% of the leaf carbon was lost.

Nitrogen content, on the other hand, perhaps decreased slightly even in phase 1, and very markedly during phase 2. In phases 3 and 4, nitrogen appeared to be remobilized slowly or not at all since the nitrogen content on a dry-weight basis only changed marginally. In total, about 80% of total leaf nitrogen (60% on a dry-weight basis) was withdrawn during autumn senescence. Phosphorus content also decreased by about 80% on a per leaf basis (60% on a dry-weight basis) during the autumn, although the phosphorus content started to decrease in phase 1 even before September 11 (Fig. 7B) and leveled out in phases 3 and 4. The remobilization of 80% of phosphorus could be compared with the breakdown of adenylates, which was 90% on leaf basis. Not surprisingly, phosphates bound to other complexes are less readily remobilized than those of the adenylate pool. Sulfur remobilization was less efficient, but still significant: the sulfur content decreased in parallel with the decrease in

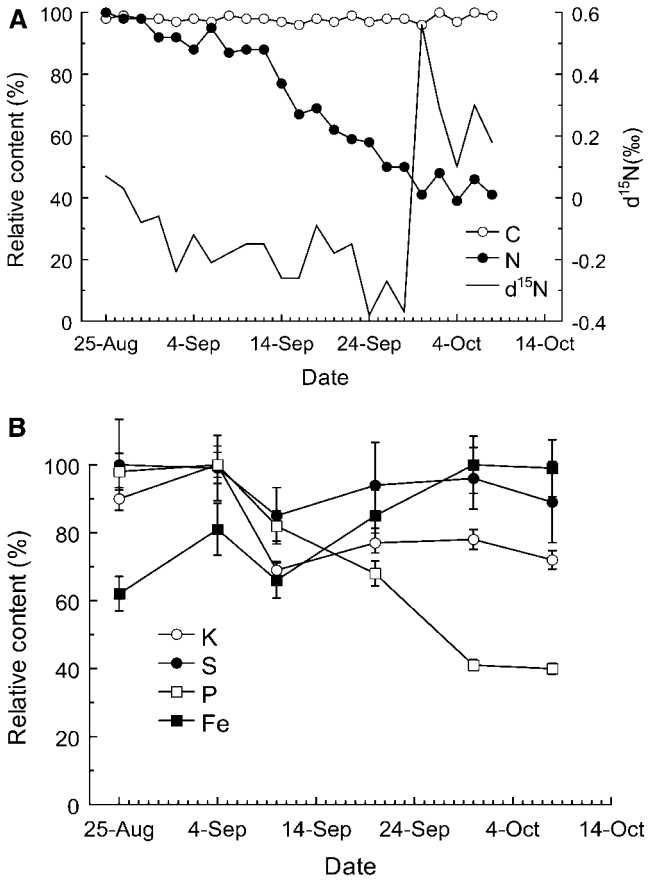

Figure 7. Elemental composition of aspen autumn leaves. A, Changes in relative contents (%) of carbon and nitrogen and in $\delta^{15}N$ values (‰). B, Changes in relative content (%) of potassium, sulfur, phosphorus, and iron. Each point is the mean value of three measurements (±SD × 2).

leaf dry weight; and the level on a dry-weight basis decreased by about 10%, showing that about 50% of the total leaf sulfur was remobilized. Potassium was also remobilized, mainly during a short time interval immediately before entry into phase 2. In phases 2 and 3, potassium levels did not change significantly on a dry-weight basis. On a dry-weight basis, the iron levels increased by about 60%. This corresponds to only about 20% of the leaf iron being remobilized. Overall, remobilization of leaf nutrients, in particular nitrogen, was efficient.

Unexpectedly, we noticed a rapid change in the $\delta^{15}N$ values of cellular content, which decreased slowly throughout phase I, showing that macromolecules with high $\delta^{15}N$ values had a slightly higher catabolic rate than the average nitrogenous molecules. This was probably because different fractions of leaf proteins had slightly different $\delta^{15}N$ values, due to differences in their biosynthetic history, and the degradation of the different fractions was not equally efficient. However, there was a very dramatic change in $\delta^{15}N$ between September 28 and September 30 (transition to phase 3), and they decreased slowly but stayed high throughout phases 3 and 4.

Chloroplasts Are Degraded and Dissolved, But a Few Plastids Remain Intact and Retain Their Chlorophyll

In order to follow the autumn senescence process in the cells ultrastructurally, we studied the leaf cells and organelles using transmission electron microscopy (TEM; Fig. 8A). Representative electron micrographs from five dates were chosen to illustrate the cellular changes during senescence, depicting the ultrastructure of both whole cells (left) and chloroplasts (right). At the end of phase 1 (September 8), the mesophyll cells had a normal shape, were lined with chloroplasts and mitochondria, and both the cytoplasm and vacuole were intact (Fig. 8A). The chloroplasts had a defined shape and structure (Fig. 8B), with plenty of starch granules and visible grana stacks. However, even at this stage the chloroplasts contained many plastoglobuli. In the middle of phase 2 (September 18), the chlorophyll concentration had decreased by 50%. Many cells (Fig. 8C) and chloroplasts (Fig. 8D) appeared almost unchanged, but the amount of electron-dense material in the cells had decreased, on average. The tonoplast was less defined and unknown structures (small vesicles) could be seen. In some cells, the chloroplasts had lost their internal membrane structure, whereas in other cells they appeared active with starch grains. There were clear differences between different sections of the leaf, which was not surprising since patchiness was also apparent at the macroscopic level at this stage, many leaves having a yellow-green mosaic pattern (Fig. 1A).

At the end of phase 2 (September 25, when 25% of the initial chlorophyll remained), many cells had lost most of their electron-dense material (Fig. 8E). Chloroplast deterioration started to be frequent (Fig. 8F),

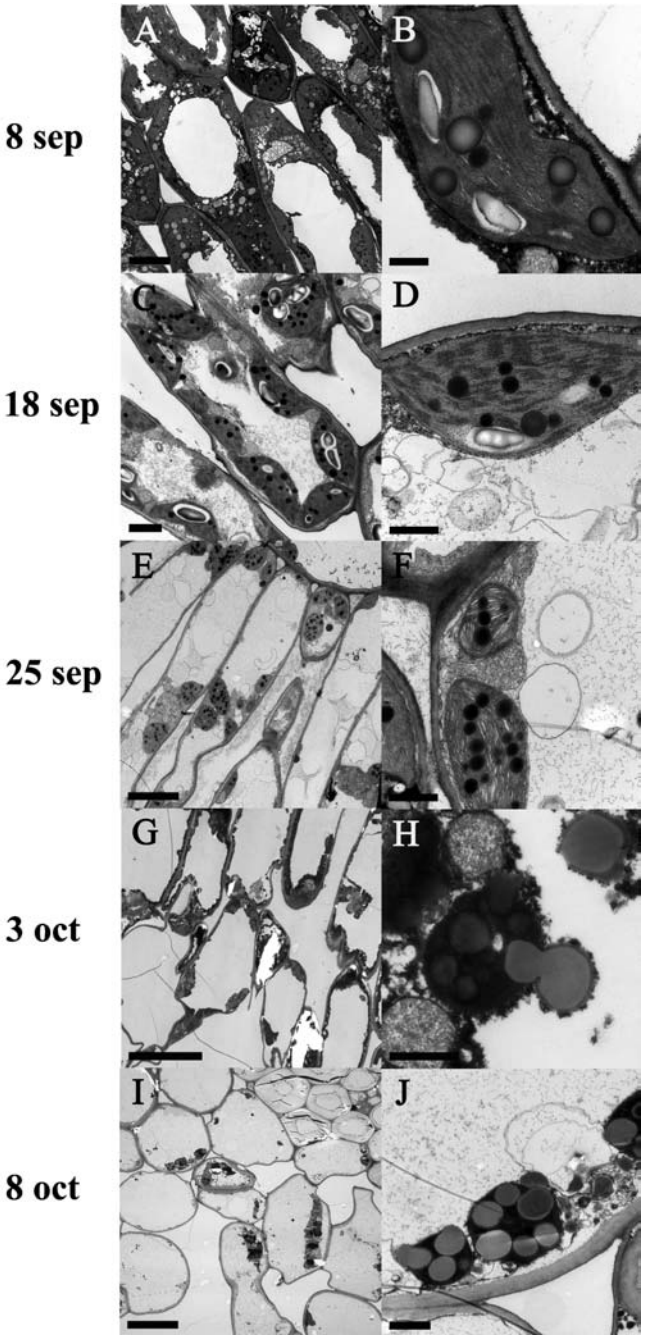

Figure 8. Changes in the ultrastructure of mesophyll cells during the autumn. Structural changes in cells (A, C, E, G, I) and chloroplasts (B, D, F, H, J) at five time points of autumn senescence (see Fig. 10). Size bars correspond to 10 μm (G and I), 5 μm (A and E), 2 μm (C), 1 μm (D, F, H, and J), and 0.5 μm (B). sep, September; oct, October.

manifested as a loss of starch granules, increases in the number and size of plastoglobuli, and loss of normal thylakoid membrane structure (replaced by swellings of the lamellae). Many small vesicle-like structures were present (Fig. 8F), apparently not located in the cytoplasm but in the vacuolar space, which gradually became less structured but more electron dense than in phase 1. Cells at this stage seemed to contain a lower

number of chloroplasts and mitochondria and the cytoplasm was confined to small areas along the walls. Some cells appeared almost empty, sometimes situated adjacent to cells containing chloroplasts. No cells seemed to contain plastids in different developmental stages, i.e. both chloroplasts and gerontoplasts, but every cell seemed to be fully synchronized in terms of plastid development.

In late phase 3, on October 3 (when less than 5% of the initial chlorophyll remained), the cells in the electron micrographs looked even less structured, and any remaining cytoplasm and plastids were aggregating in the corners of some cells, whereas others appeared empty (Fig. 8G). The plastids found were swollen gerontoplasts, consisting mainly of large plastoglobuli, but some mitochondria and a few nuclei still looked intact (Fig. 8H). Some plastoglobuli were also starting to extrude in blobs from the gerontoplasts, perhaps corresponding to the mass exodus that has been reported from soybean gerontoplasts (Guiamet et al., 1999), and the cytoplasmic regions were heterogeneous and contained vesicle-like structures. In phase 4, finally, most cells appeared empty and dead (Fig. 8I), although some still contained gerontoplasts filled with plastoglobuli, and some mitochondria-like structures were found (Fig. 8J).

We were intrigued by the findings that the chlorophyll content of phase 3 leaves was about 5% of that of phase 1 leaves (although they appeared yellow), and that some leaves yielded a detectable F_v/F_m ratio. To get a better overview of the chlorophyll compartmentalization in late phase 3 leaves, we scanned leaves sampled on October 6 using confocal microscopy and recorded chlorophyll autofluorescence.

Figure 9 shows a sectioned leaf at two different magnifications. The remaining chlorophyll was found in plastids, or at least organelle-like structures, throughout the entire mesophyll (both palisade cells and spongy mesophyll), but more frequently close to the veins. Some cells were clearly devoid of plastids, but some contained one or a few chlorophyll-containing plastids, sometimes located in opposite corners of the cell. This and the TEM micrographs show that, even at this very late stage of senescence, some cells contained chlorophyll-containing plastids, perhaps performing a metabolic function.

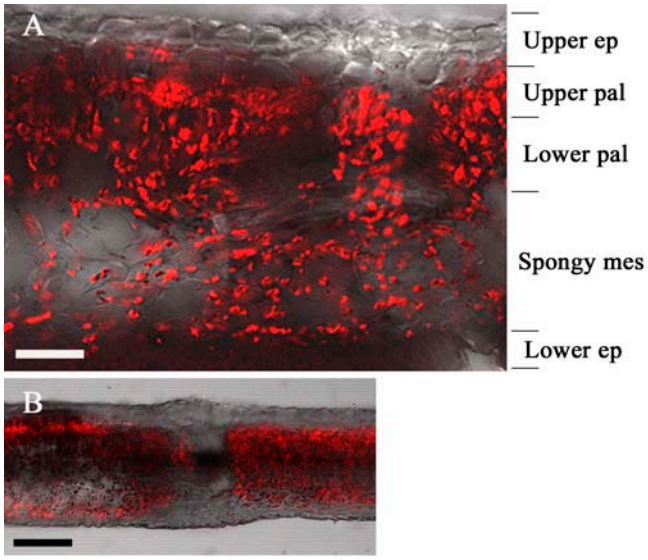

Figure 9. Chlorophyll autofluorescence (red) measured using confocal microscopy in a phase 4 leaf (October 6). A, Mesophyll tissue. B, Leaf segment. Size bars correspond to 50 μm (top) and 100 μm (bottom). ep, Epidermis; pal, palisade; mes, mesophyll.

DISCUSSION

The tree we are studying has several million leaves. Each leaf contains about 30 million cells and each cell on average around 40 chloroplasts. The magnificent appearance of autumn senescence is the result of the synchronized and apparently tightly controlled conversion of these $\approx 10^{15}$ chloroplasts to gerontoplasts and the subsequent degradation of the majority of the cell organelles, including the gerontoplasts. We have followed this process by measuring pigments, key metabolites like ATP and Suc, macronutrients, photosynthesis, and cell and organelle integrity throughout the whole process. The data generated can be compiled to create a cellular timetable of autumn senescence (Fig. 10) in which the senescence process is divided into four temporal phases to emphasize key events, the first detailed description of the cellular events that occur during autumn senescence. This is, of course, not an absolute and generic timetable, since different tree species follow different patterns and even within a single species there is significant variation in, for example, the onset of the process. Factors like nutrient status also affect the initiation of the process. Nevertheless, we believe that studies of a single tree in a single year have general significance and are informative about the order of events involved. Reliable data on the sequence of events are required for a mechanistic understanding of the process and to allow detailed studies to address variation between species and individuals.

By constructing the cellular timetable of autumn senescence for an aspen tree, we believe that we have obtained novel insights into some of the major unresolved questions concerning autumn senescence. We have shown that the entry into phase 2 was triggered by the photoperiod and that temperature (and light) had little or no effect on the onset of senescence. The autumn senescence process, once initiated by environmental factors, seems to be a tightly controlled developmental program that is not significantly altered by environmental factors. Chlorophyll was degraded at a fairly constant rate, although weather conditions fluctuated (and differed between years). If photooxidation of pigments had been a major determinant, the degradation rate would have been increased by cold clear days (like September 19 and 20), for example, which was not the case (Kukavica and Jovanovic, 2004).

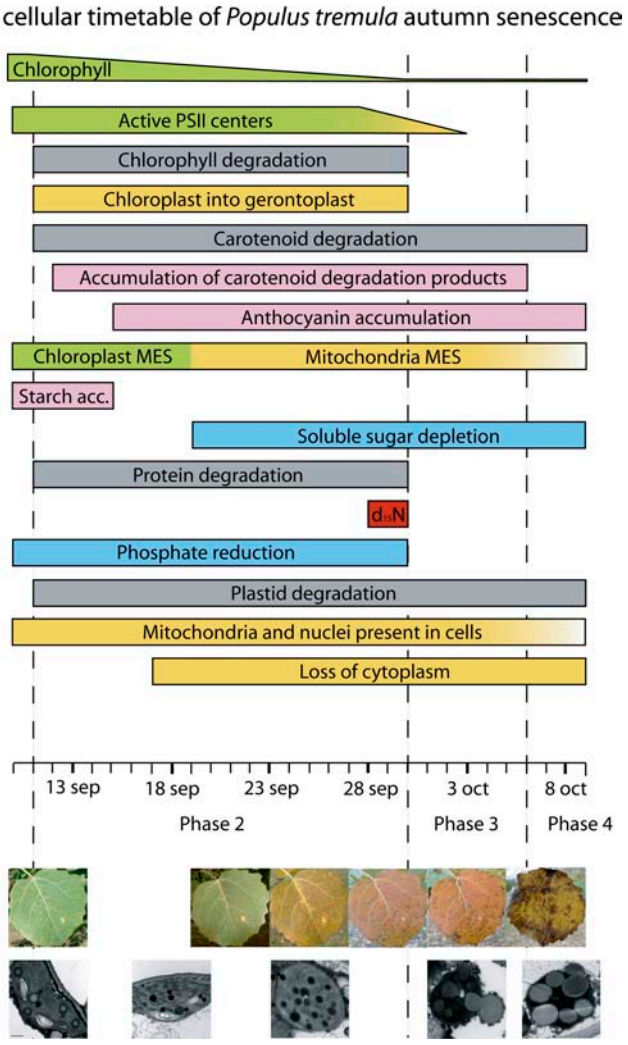

Figure 10. A cellular timetable of *Populus tremula* autumn senescence, compiled from the data presented in this paper. "Loss of cytoplasm" denotes the combination of two events, cytoplasm degradation and vacuolar burst. MES, Main energy source.

Chlorophyll was degraded at a constant rate, and carotenoid catabolites started to appear after September 12, before gerontoplast formation was observed. Photooxidative stress, on the other hand, seemed to be the major determinant of anthocyanin accumulation, and anthocyanin levels changed dynamically depending on the weather. The stimulation of autumn colors by cold weather seemed therefore to be solely due to effects on anthocyanin accumulation. Anthocyanin accumulation seemed to correlate better with excess light (high light and cold) than with either high light or cold alone, consistent with the hypothesis that anthocyanins have a photoprotective role. The large fluctuations in anthocyanin content showed that anthocyanins were rapidly metabolized right up to leaf abscission. Anthocyanin catabolism may be light regulated or, alternatively, regulated anabolism in aspen leaves could compete with a seemingly unregulated catabolism, similar to the regulation of the xanthophyll cycle. Interestingly, leaves in phase 1 did not seem to be as competent to respond to the stimuli that later led to anthocyanin accumulation. September 1 and 2 were as cold as September 19, and the light was brighter, but no anthocyanin accumulation was induced at this time. These observations, together with the fact that damaged PSII centers were also repaired during the first week of September, demonstrate that the leaves at this point were not yet ready to enter the senescence process. This is consistent with our previous suggestion that a peak in transcriptional activity in the first week of September coincides with a developmental switch reprogramming the leaf to senescence (Andersson et al., 2004).

On September 11, the degradation process started (phase 2). This date for entry into phase 2 was the same over several years, with different weather conditions, and is therefore likely to be controlled by the photoperiod. A senescence signal triggered the cells to degrade their photosystems (and chlorophyll), thereby releasing carotenoids, some of which (lutein and β-carotene) were degraded, whereas others (particularly neoxanthin and violaxanthin) were esterified and accumulated in the plastoglobuli. This process seemed, within each plastid, to be very rapid, since the F_v/F_m ratios stayed high and constant until very late in the process. If large proportions of PSII centers present in the chloroplasts had been undergoing degradation processes, this would probably have been manifested by reductions in the overall F_v/F_m values. Apparently, therefore, although total photosynthetic capacity decreases along with the chlorophyll degradation, the photosynthetic parameters of the remaining PSII centers stay constant until very late in the process. We interpret these data as indicating that the transition from a photosynthetically fully active chloroplast to a gerontoplast is very rapid, and these data are consistent with a model in which all of the chloroplasts in a given cell undergo the transition simultaneously, while a neighboring cell could be in a different developmental stage. Changes in the chlorophyll a/b ratio showed that the degradation of antenna chlorophylls at the end of phase 2 is slower than that of chlorophyll a in the reaction centers. This resembles somewhat the situation in some stay-green mutants, where the remaining chlorophyll in otherwise senescing leaves is bound to light-harvesting complex II (Hilditch et al., 1986). The adaptive benefit of keeping the antenna and dismantling the reaction centers is not clear, but has been demonstrated during leaf senescence in other species (Wolf, 1956; Lichtenthaler, 1987). In parallel with the chloroplast degeneration, expression of the photosynthetic genes was strongly repressed (Andersson et al., 2004). After entry into phase 2, the cytoplasm started to be degraded, eventually making it difficult to distinguish cytoplasm from the vacuole. We could detect small vesicles, perhaps similar to the vesicles reported by Otegui et al. (2005), nuclei gradually disappeared, and the cells seem to largely stop

synthesizing protein at this point; at least we have not been able to extract high-quality RNA from the leaves after September 24 (Andersson et al., 2004).

During the leaf senescence process, most of the nitrogen and phosphorus was retrieved. The relative content of carbon was not changed (Fig. 7A), but since the leaf dry weight decreased (Fig. 2B) some carbon may also be retained. This massive remobilization requires, of course, the involvement of many catabolic enzymes, and genes encoding various types of proteases, lipases, and enzymes involved in nucleotide metabolism, glyconeogenesis, and nitrogen remobilization (inter alia) are either induced, or continue to be expressed, in the autumn leaves (Bhalerao et al., 2003; Andersson et al., 2004; Terce-Laforgue et al., 2004).

Senescence is an energy-requiring process in which ATP is needed for degradation, recycling, and transporting nutrients out of the cell. As photosynthesis declines in phase 2, mitochondrial respiration needs to take over to provide the cell with the energy required for these processes. Thus, a significant fraction of the carbon lost from the leaf is likely to be due to respiration and not to be retrieved. We did not measure respiration during the process, but it is clear that mitochondria stay active throughout senescence since intact mitochondria could be seen in electron microscopy images late into the process. Genes encoding components of mitochondrial electron transport also continue to be expressed throughout the period (Andersson et al., 2004). By monitoring changes in the levels of key metabolites (ATP/ADP ratios and pool size, starch, and various soluble sugars), we found evidence indicating that a shift in the main energy sources of the cell from chloroplasts to mitochondria probably occurred around September 19.

On September 30, the degradation process was largely complete and the leaves had entered phase 3. It is likely that this transition coincides with the formation of the protection and separation layers in the petiole, which blocks further phloem transport out of the leaf. The changes after this date were minor, and since leaves started to be shed and individual leaves in which the process was somewhat retarded were more likely to be withheld, this may explain the weakness of some trends in the data and suggests that our separation between phase 3 and phase 4 may be artificial. Some cells seemed at this stage to be empty (and probably dead), whereas many contained nuclei, mitochondria, and a small number of gerontoplasts. Vacuoles and cytoplasm could not be distinguished from each other. The dynamic changes in variables, such as the levels of xanthophyll cycle pigments and anthocyanins, and the presence of a few active photosystems during phase 3 suggest that a significant fraction of the cells were still alive and metabolically active. Many of these cells contained chlorophyll-containing plastids, and the residual chlorophyll level was approximately 5% of the phase 1 levels, but only a small fraction of this remaining chlorophyll seemed to be photosynthetically active, and this activity was lost during phase 3. The residual chlorophyll is likely to be localized in the plastoglobuli, where the high amounts of carotenoids could serve as efficient quenchers of excess excitation energy.

The sudden shift in the nitrogen isotope ratio at the very end of phase 2 (September 29) was an unexpected finding. Changes in the nitrogen isotope ratios of senescing pine needles have previously been reported, and three hypotheses have been put forward to explain them (Näsholm, 1994). Since two of the hypotheses (discrimination against $\delta^{15}N$ in the process of nitrogen translocation and the possibility that nitrogen pools that are not degraded, like cell wall proteins, have relatively high $\delta^{15}N$ values) are incompatible with such a dramatic shift in the ratio; the third hypothesis, the emission of nitrogen-containing volatile compounds (low in $\delta^{15}N$), may be the best explanation for our findings. Emission of volatiles from senescing leaves has been reported (Husted et al., 1996), and our finding that the sudden change in isotope ratio coincided with the transition from phase 2 to phase 3, and probably with the termination of phloem transport out of the leaves, is compatible with a hypothesis that nitrogen-containing compounds may accumulate in the leaf to toxic levels, since protein catabolism probably continues so ammonia may be released to reduce the amount of excess nitrogen. It is possible that this sudden change in $\delta^{15}N$ is a signature of the end of the remobilization process in autumn leaves.

There are two possible explanations for the retention of some cells and organelles throughout phase 3. We believe that, by the end of phase 2, the tree has gone through the essential steps in the senescence process, enabling nutrient retrieval to occur, and that a separation layer has formed, so very little further remobilization is possible. However, if there are leaves that are still able to export some material, catabolic activities will be required for the remobilization. The accumulation of plastids, probably an indicator of living cells, closer to the veins is compatible with the hypothesis that, during phase 3 (and 4), the cells located far away from the veins may die, but that cells closer to the veins could participate in the degradation of macromolecules. The tree may be prepared for the winter by the end of phase 2, but if no frost hard enough to kill the cells occurs, the tree gets a bonus phase in which a small fraction of the remaining nutrients could perhaps be remobilized. The second possible explanation is that if the leaf cells have functionally separated from the trunk, their fate will not influence the rest of the tree so they may live or die without any particular adaptive benefit. In any case, these data are compatible with the concept that leaf senescence and cell death are to some extent antagonistic processes.

Our data show that anthocyanin accumulation is likely to have a role in photoprotection, as suggested by several authors (Feild et al., 2001; Hoch et al., 2003). In the ecological literature, there has recently been speculation about a possible connection between autumn senescence and insect herbivory (Archetti, 2000;

Hagen et al., 2003), assuming that autumn colors may be an honest, or dishonest, signal to insects about the status of the tree. Strong arguments against this, from a physiological perspective, have been put forward by Ougham et al. (2005) and, given the fact that accumulation and degradation of pigments in autumn leaves can be explained by obvious physiological and developmental factors, we think that there is unlikely to be a direct link between autumn colors and herbivore preference, and instead the phenomena are highly correlated because both are highly dependent on the nitrogen status of the tree.

Is there a point of no return in autumn senescence of aspen leaves? To our knowledge, this question has not been experimentally addressed, but considering the events that we have followed here, one could speculate that the reprogramming of gene expression late in phase 1 could be an irreversible process or, alternatively, that the leaves could re-green if the whole tree is exposed to a longer photoperiod. However, it is doubtful that such a capability would have adaptive significance in nature, since trees will never experience an increase in the photoperiod during the autumn. However, we now have the tools to also address this question.

By detailed studies of a tree during autumn senescence, we believe that we have obtained novel insights into the fundamental questions posed in the introduction. The conversion of chloroplasts to gerontoplasts is driven by developmental factors, the nutrients are largely remobilized over a period of 18 d (Sept 11 to Sept 29, phase 2), and, in the middle of this phase, mitochondria take over as the main providers of energy for the process. During this phase, plastids are converted to gerontoplasts and many cells die, but some survive and retain their gerontoplasts, mitochondria, and perhaps also nuclei until the leaves abscise, up to 1 week later.

Finally, the photoperiod seems to be the sole trigger for the onset of autumn senescence in aspen, and the signal transduction chain between the photoreceptor phytochrome and the downstream genes that execute the senescence program can now be investigated with molecular and genomic tools.

MATERIALS AND METHODS

Leaf Material

Leaves were sampled every day at 11 to 11:30 AM between August 25 and October 9, 2003, from a free-growing aspen tree (*Populus tremula*) on the Umeå University campus (see Bhalerao et al., 2003). Five leaves were picked at each time point, pooled, and ground in liquid nitrogen. Once or twice a week, two single leaves were picked for fixation, embedding, and TEM. Fresh weight and dry weight were measured and mean leaf weight was calculated.

Spectroscopic Measurements

Chlorophyll

Triplicates of about 30 mg of ground leaf material (a mixture of five leaves for each date) were extracted in 80% aqueous acetone solution buffered with 25 mM HEPES. The extract was analyzed for absorbance at wavelengths of 646.6, 663.6, and 750.0 nm using a Biochrom 4060 spectrophotometer (Pharmacia LKB Biochrom). The concentrations of chlorophyll *a* and chlorophyll *b* were then calculated using the equations of Porra et al. (1989).

Anthocyanins

Duplicates of about 40 mg of ground leaf material (a mixture of five leaves for each date) were extracted with 85% aqueous acetone and 1% HCl (85:15) at 4°C. After phase separation with diethylether to remove chlorophylls, the aqueous extract was analyzed for absorbance at wavelengths of 535.0, 650.0, and 750.0 nm using a Biochrom 4060 spectrophotometer (Pharmacia LKB Biochrom). Anthocyanin concentrations were then calculated using the extinction coefficient ($E^{1\%}_{1cm}$=98.2 at λ = 535 nm) according to Do and Cormier (1991).

Pigment Analysis by HPLC

Carotenoids were extracted with 96% ethanol and quantified according to Król et al. (1995). Pigments were detected by measuring the absorbance at 440 nm. Retention times and response factors for chlorophyll *a*, chlorophyll *b*, lutein, β-carotene, neoxanthin, violaxanthin, antheraxanthin, and zeaxanthin were previously determined (Król et al., 1995). Analysis was performed using the chromatograph's software (System Gold; Beckham Instruments). To minimize instrumental bias, samples were run in random order. The epoxidation state of xanthophyll cycle pigments was calculated according to the formula (V + 1/2A)/(V + A + Z).

Several unidentified peaks were found in the chromatograms in extracts from the later stages of senescence. We assumed that some of these pigments may have been carotenoid esters, so we saponified extracts from late phase 3 (October 3) by adding 25 μL of 20% KOH to the extracts, then incubating the mixtures in darkness on ice for 18 h. Saponified extracts were filtered again before the HPLC analysis. The concentrations of neoxanthin, violaxanthin, and antheraxanthin increased by about 50% in the saponified extracts, indicating that these carotenoids were released from esters upon saponification. The amounts of zeaxanthin, lutein, β-carotene, chlorophyll *a*, and chlorophyll *b* changed only marginally. Thus, the results suggest that esters of neoxanthin, violaxanthin, and antheraxanthin were present in the autumn leaves.

Two of the pigments (with retention times of 6.2 and 6.5 min, respectively) were not affected by saponification; the first pigment only marginally accumulated during senescence and is sometimes present in leaves of other plants (data not shown), while the second was not detected until September 19 and accumulated thereafter. The first pigment had a similar absorbance spectrum to violaxanthin (peaks at 443 and 472 nm in our system), and the second pigment had peaks at 448 and 473 nm. These are denoted carotenoids X and Y (CX and CY). Two pigments (retention times 8.2 and 8.3 min, respectively) had absorbance peaks at 438 and 467 nm (similar to neoxanthin) and disappeared upon saponification. These pigments were tentatively identified as neoxanthin esters (NE1 and NE2). Another pigment with a retention time of 8.5 min also disappeared upon saponification but had an absorbance spectrum resembling that of violaxanthin, with peaks at 443 and 471 nm, so this pigment could represent a violaxanthin ester (VE1). The peak with a retention time of 8.9 min was a nonresolved doublet that disappeared in the saponified extract. The absorbance spectrum of this peak most resembled that of neoxanthin, so we denoted it NE3, despite the fact that it most likely corresponds to two different carotenoid esters. A doublet appeared at retention times of 9.4 and 9.6. These pigments disappeared upon saponification. The absorbance spectra of the leading and trailing peaks in the doublet resembled those of neoxanthin and violaxanthin, respectively. These peaks were denoted NE4 and VE2, respectively. Finally, the peak with the largest area (retention time 10 min) had a spectrum resembling that of violaxanthin, but this pigment was stable during saponification. We tentatively denoted this pigment VX.

These pigments have not been rigorously identified, so the assignments were made purely for practical reasons and are only tentative. For example, the increase in the amount of antheraxanthin after saponification indicates that antheraxanthin esters were present, but none of the peaks have been assigned as antheraxanthin esters. In addition to the pigments discussed here, several minor unassigned peaks (with typical carotenoid absorbance spectra) also appeared in the chromatograms.

ATP and Sugar Measurements

About 20 mg of ground leaf material (a mixture of five leaves for each date) was extracted in 3% TCA. ATP was determined by the firefly luciferase

method (Gardeström and Wigge, 1988). ADP was measured after pyruvate kinase-catalyzed conversion to ATP (Roche Diagnostics).

Soluble sugars (Suc, Glc, and Fru) and starch were measured in the soluble and residual fractions of ethanol-water extracts according to Stitt et al. (1989).

TEM

Ten pieces, each with a surface area of about 1.5 mm^2, were cut from each leaf and infiltrated under mild vacuum for 15 min in a 3% (w/v) glutaraldehyde/0.1 M phosphate buffer (pH 7.2), then incubated for 4 h at room temperature (primary fixation), washed with phosphate buffer, and changed to 2% (w/v) osmium tetroxide for 2 h (secondary fixation). After washing in distilled water, the samples were dehydrated in a graded ethanol series, transferred to propyleneoxide for 3×10 min, and then epoxyresin (TAAB 812; TAAB Laboratories) was added dropwise every 10 min until the resin concentration reached approximately 10% (v/v). The samples were then left on a rotator overnight. The next day, the resin was sequentially changed every 4 h to 25%, 50%, and finally 75%, then incubated at 75% overnight, after which they were kept in 100% resin for one-half day and then embedded on silicon frames and incubated at 60°C for 1 d. After embedding, the samples were cut with a diamond knife and transferred to Formvar-coated 200 mesh copper grids. The grids were stained with 3% aqueous uranyl acetate for 30 min and with lead citrate for 10 min before being examined under a JEOL JEM 1230 transmission electron microscope operated at 80 kV.

Elemental Analysis

N%, C%, $\delta^{13}C$, and $\delta^{15}N$ values of the pooled leaf samples were measured according to Ohlsson and Wallmark (1999), using a continuous flow isotope ratio mass spectrometer (20–20 Stable Isotope Analyzer; Europa Scientific) interfaced with an elemental analyzer unit (ANCA-NT system, solid/liquid preparation module; Europa Scientific).

Iron, sulfur, phosphorus, and potassium concentrations of the pooled leaf samples were measured according to Emteryd (2003), using an inductively coupled plasma mass spectrometer (Elan 6100; Perkin-Elmer).

Fluorescence Measurements

Fluorescence measurements were performed at approximately noon roughly every second or third day on the same four leaves using a portable plant stress meter (version 2.12; Biomonitor S.C.I.). Prior to fluorescence measurements, the leaves were dark adapted for at least 40 min using clamp cuvettes, according to Öquist and Wass (1988).

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